INSECT-RESISTANT PLANTS

The present invention relates to the fields of genetic engineering, biochemistry and plant transformation. More particularly, the present invention is directed toward transformation of plant cells to express a chimeric gene encoding a protein toxic to Coleopteran insects.

Bacillus thuringiensis (*B.t.*) is a spore forming soil bacterium which is known for its ability to produce a parasporal crystal protein which is toxic to a wide variety of insects. Most strains are active against Lepidopteran insects (moths and butterflies) and a few are reported to have activity against Dipteran insects (mosquitoes and flies, see Aronson et al. 1986). Toxin genes from a variety of these strains have been cloned and the toxins have been expressed in heterologous hosts (Schnepf et al., 1981; Klier et al., 1982). In recent years, *B.t.* var. *tenebrionis* (*B.t.t.*, Krieg et al., 1983; Krieg et al., 1984) and *B.t.* var. *san diego* (*B.t.sd.*, Herrnstadt et al., 1986) strains have been identified as having activity against Coleopteran insects. The toxin gene from *B.t.sd.* has been cloned, but the toxin produced in *E. coli* was reported to be a larger size than the toxin from *B.t.sd.* crystals, and activity of this recombinant *B.t.sd.* toxin was implied to be weak.

Insects susceptible to the action of the protein toxin of Coleopteran-type *Bacillus thuringiensis* bacteria include, but are not limited to, Colorado potato beetle (*Leptinotarsa decemlineata*), boll weevil (*Anthonomus grandis*), yellow mealworm (*Tenebrio molitor*), elm leaf beetle (*Pyrrhalta luteola*) and Southern corn rootworm (*Diabrotica undecimpunctata howardi*).

Therefore, the potential for genetically engineered plants which exhibit toxicity or tolerance toward Coleopteran insects was foreseen if such plants could be transformed to express a Coleopteran-type toxin at a insecticidally-effective level. Agronomically important crops which are affected by Coleopteran insects include alfalfa, cotton, maize, potato, rape (canola), rice, tobacco, tomato, sugar beet and sunflower.

Although certain chimeric genes have been expressed in transformed plant cells and plants, such expression is by no means straight forward. Specifically, the expression of Lepidopteran-type *B.t.* toxin proteins has been particularly problematic. It has now been found that the teachings of the art with respect to expression of Lepidopteran-type *B.t.* toxin protein in plants do not extend to Coleopteran-type *B.t.* toxin protein. These findings are directly contrary to the prior teachings which suggested that one would employ the same genetic manipulations to obtain useful expression of such toxins in transformed plants.

In accordance with one aspect of the present invention, there has been provided a method for producing genetically transformed plants which exhibit toxicity toward Coleopteran insects, comprising the steps of:

(a) inserting into the genome of a plant cell susceptible to attack by Coleopteran insects a chimeric gene comprising:

i) a promoter which functions in plant cells to cause production of RNA;

ii)   a DNA sequence that causes the production of a RNA sequence encoding a Coleopteran-type toxin protein of *Bacillus thuringiensis*; and

iii) a 3' non-translated DNA sequence which functions in plant cells to cause the addition of poly- adenylate nucleotides to the 3' end of the RNA sequence;

(b) obtaining transformed plant cells, and

(c) regenerating from the transformed plant cells genetically transformed plants exhibiting resistance to Coleopteran insects.

In accordance with another aspect of the present invention, there has been provided a chimeric plant gene comprising in sequence:

(a) a promoter which functions in plant cells to cause the production of RNA;

(b) a DNA sequence that causes the produc- tion of a RNA sequence encoding a Coleopteran-type toxin protein of *Bacillus thuringiensis*; and

(c) a 3' non-translated region which func- tions in plant cells to cause the addi- tion of polyadenylate nucleotides to the 3' end of the RNA sequence.

There has also been provided, in accordance with another aspect of the present invention, bacterial cells, transformed plant cells and plant transforma- tion vectors that contain, respectively, DNA comprised of the above-mentioned elements (a), (b) and (c).

In accordance with yet another aspect of the present invention, a differentiated plant has been provided that comprises transformed plant cells, as described above, which exhibit toxicity to Coleopteran insects. The present invention also contemplates seeds which produce the above-described transformed plants.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the DNA probes used for isolation of the *B.t.t.* toxin gene.

Figure 2 shows the steps employed in the preparation of plasmid pMON5432.

Figure 3 shows the orientation of the 3.0 kb HindIII fragment encoding the toxin gene in pMON5420 and pMON5421 with respect to the multilinker of pUC119.

Figure 4 shows the strategy utilized for sequencing of the *B.t.t.* toxin gene contained in pMON5420 and pMON5421.

Figure 5 shows the DNA sequence and location of restriction sites for the 1932 bp ORF of the *B.t.t.* gene encoding the 644 amino acid toxin protein.

Figure 6 shows the bands observed for *B.t.t.* toxin following SDS-PAGE analysis.

Figure 7 shows the N-termini of proteins expressed from the *B.t.t.* toxin gene or proteolytically produced *in vivo* in *B.t.t.*

Figure 8 represents the altered *B.t.t.* genes used to analyze the criticality of the C-terminal portion of the toxin.

Figure 9 represents the altered *B.t.t.* genes used to analyze the criticality of the N-terminal portion of the toxin.

Figure 10 shows the deletions produced in evaluation of *B.t.t.* toxin protein mutants.

Figure 11 shows the steps employed in preparation of plasmids pMON9758, pMON9754 and pMON9753.

Figure 12 shows the steps employed in preparation of plasmid pMON9791.

Figure 13 shows the steps employed in preparation of plasmid pMON9792.

Figure 14 shows a plasmid map for plant transformation cassette vector pMON893.

Figure 15 shows the steps employed in preparation of plasmid pMON9741.

Figure 16 shows the steps employed in the preparation of plasmid pMON5436.

Figure 17 illustrates the elements comprising the T-DNA region of disarmed *Agrobacterium* ACO.

Figure 18 shows the DNA sequence for the enhanced CaMV35S promoter.

STATEMENT OF THE INVENTION

The present invention provides a method for transforming plants to exhibit toxicity toward susceptible Coleopteran insects. More particularly, the present invention provides transgenic plants which express the Coleopteran-type toxin protein of *Bacillus thuringiensis* at an insecticidal level.

In one aspect, the present invention comprises chimeric genes which function in plants and produce transgenic plants which exhibit toxicity toward susceptible Coleopteran insects. The expression of a plant gene which exists as double-stranded DNA involves the transcription of one strand of the

DNA by RNA polymerase to produce messenger RNA (mRNA), and processing of the mRNA primary transcript inside the nucleus. This processing involves a 3' non-translated region which adds polyadenylate nucleotides to the 3' end of the mRNA.

Transcription of DNA to produce mRNA is regulated by a region of DNA usually referred to as the "promoter." The promoter region contains a sequence of nucleotides which signals RNA polymerase to associate with the DNA, and initiate the production of a mRNA transcript using the DNA strand downstream from the promoter as a template to make a corresponding strand of RNA.

A number of promoters which are active in plant cells have been described in the literature. These include the nopaline synthase (NOS), octopine synthase (OCS) and mannopine synthase (MAS) promoters which are carried on tumor-inducing plasmids of *Agrobacterium tumefaciens*, the cauliflower mosaic virus (CaMV) 19S and 35S promoters, and the light-inducible promoter from the small subunit of ribulose bis-phosphate carboxylase (ssRUBISCO, a very abundant plant polypeptide). These types of promoters have been used to create various types of DNA constructs which have been expressed in plants; see e.g., PCT publication WO 84/02913 (Rogers et al., Monsanto).

Promoters which are known or are found to cause production of a mRNA transcript in plant cells can be used in the present invention. Suitable promoters may include both those which are derived from a gene which is naturally expressed in plants and synthetic promoter sequences which may include redundant or heterologous enhancer sequences. The promoter selected should be capable of causing sufficient

expression to result in the production of an effective amount of toxin protein to render the plant toxic to Coleopteran insects. Those skilled in the art recognize that the amount of toxin protein needed to induce the desired toxicity may vary with the particular Coleopteran insects to be protected against. Accordingly, while the CaMV35S, ssRUBISCO and MAS promoters are preferred, it should be understood that these promoters may not be optimal promoters for all embodiments of the present invention.

The mRNA produced by the chimeric gene also contains a 5' non-translated leader sequence. This sequence may be derived from the particular promoter selected such as the CaMV35S, ssRUBISCO or MAS promoters. The 5' non-translated region may also be obtained from other suitable eukaryotic genes or a synthetic gene sequence. Those skilled in the art recognize that the requisite functionality of the 5' non-translated leader sequence is the enhancement of the binding of the mRNA transcript to the ribosomes of the plant cell to enhance translation of the mRNA in production of the encoded protein.

The chimeric gene also contains a structural coding sequence which encodes the Coleopteran-type toxin protein of *Bacillus thuringiensis* or an insecticidally-active fragment thereof. Exemplary sources of such structural coding sequences are *B. t. tenebronis* and *B. t. san diego*. Accordingly, in exemplary embodiments the present invention provides a structural coding sequence from *Bacillus thuringiensis* var. *tenebrionis* and insecticidally-active fragments thereof. Those skilled in the art will recognize that other structural coding sequence substantially homologous to the toxin coding sequence of *B.t.t.* can be utilized

following the teachings described herein and are, therefore, within the scope of this invention.

The 3' non-translated region contains a polyadenylation signal which functions in plants to cause the addition of polyadenylate nucleotides to the 3' end of the RNA. Examples of suitable 3' regions are (1) the 3' transcribed, non-translated regions containing the polyadenylate signal of the tumor-inducing (Ti) plasmid genes of *Agrobacterium*, such as the nopaline synthase (NOS) gene, and (2) plant genes like the soybean storage protein genes and the ssRUBSICO. An example of preferred 3' regions are those from the NOS, ssRUBISCO and storage protein genes, described in greater detail in the examples below.

The Coleopteran-type toxin protein genes of the present invention are inserted into the genome of a plant by any suitable method. Suitable plant trans- formation vectors include those derived from a Ti plasmid of *Agrobacterium tumefaciens* such as those described in, e.g. EPO publication 131,620 (Rogers et al.), Herrera-Estrella 1983, Bevan 1983, Klee 1985 and EPO publication 120,516 (Schilperoort et al.). In addition to plant transformation vectors derived from the Ti or root-inducing (Ri) plasmids of *Agrobacte- rium*, alternative methods can be used to insert the Coleopteran-type toxin protein genes of this invention into plant cells. Such methods may involve, for example, liposomes, electroporation, chemicals which increase free DNA uptake, and the use of viruses or pollen as vectors. If desired, more than one gene may be inserted into the chromosomes of a plant, by methods such as repeating the transformation and selection cycle more than once.

The plant material thus modified can be assayed, for example, by Northern blotting, for the presence of Coleopteran-type toxin protein mRNA. If no toxin protein mRNA (or too low a titer) is detected, the promoter used in the chimeric gene construct is replaced with another, potentially stronger promoter and the altered construct retested. Alternately, level of toxin protein may be assayed by immunoassay such as Western blot. In many cases the most sensitive assay for toxin protein is insect bioassay.

This monitoring can be effected in whole regenerated plants. In any event, when adequate production of toxin protein mRNA is achieved, and the transformed cells (or protoplasts) have been regenerated into whole plants, the latter are screened for resistance to attack by Coleopteran insects. Choice of methodology for the regeneration step is not critical, with suitable protocols being available for hosts from *Leguminosae* (alfalfa, soybean, clover, etc.), *Umbelliferae* (carrot, celery, parsnip), *Cruciferae* (cabbage, radish, rapeseed, etc.), *Cucurbitaceae* (melons and cucumber), *Gramineae* (wheat, rice, corn, etc.), *Solanaceae* (potato, tobacco, tomato, peppers), *Malvaceae* (cotton, etc.), *Chenopodiaceae* (sugar beet, etc.) and various floral crops. See e.g. Ammirato et al. (1984).

All protein structures represented in the present specification and claims are shown in conventional format wherein the amino group at the N-terminus appears to the left and the carboxyl group at the C-terminus at the right. Likewise, amino acid nomenclature for the naturally occurring amino acids found in protein is as follows: alanine (ala;A), asparagine (Asn;N), aspartic acid (Asp;D), arginine

(Arg;R), cysteine (Cys;C), glutamic acid (Glu;E), glutamine (Gln;Q), glycine (Gly;G), histidine (His;H), isoleucine (Ile;I), leucine (Leu;L), lysine (Lys;K), methionine (Met;M), phenylalanine (Phe;F), proline (Pro;P), serine (Ser;S), threonine (Thr;T), tryptophan (Trp;W), tyrosine (Tyr;Y) and valine (Val;V).

ISOLATION OF *B. t. t.* TOXIN GENE

The *B. t. t.* gene encoding the Coleopteran-type toxin protein was isolated as described below.

Isolation of Protein Crystals

B. t. tenebrionis was grown in Trypticase Soybroth (TSB) medium for the isolation of protein crystals. In attempting to isolate intact crystals from *B. t. t.* a significant difference between these crystals and those of the Lepidopteran-type was noted. While Lepidopteran-type crystals are routinely isolated on gradients formed from Renografin, Hypaque or NaBr, it was found that *B. t. t.* crystals dissolved in these gradients media. It was found that *B. t. t.* crystals were stable in gradients of sucrose, and sucrose gradients were used for the isolation of *B. t. t.* crystals.

Isolation of *B. t. t.* Toxin from Crystals

Purified crystals were analyzed for their protein composition by SDS polyacrylamide gel electrophoresis. Results of these experiments indicated that *B. t. t.* crystals contained at least two protein components with molecular weights of approximately 68 to 70 kilodaltons (kDa) and approximately 60 kDa, respectively. The relative amounts of the components were variable from preparation to preparation. In addition,

it was suggested that the higher molecular weight
component might consist of more than a single protein.
Bernhard (1986) reported proteins of about 68 kDa and
50 kDa as components of *B.t.t.* crystals. Herrnstadt
5   et al. (1986) reported that the crystals of *B.t. san
diego* were composed of a protein of about 64 kDa. In
contrast, Lepidopteran-type *B.t.* strains such as *B.t.
kurstaki* typically contain a higher molecular weight
protein of 130 kDa to 140 kDa. This result indicates
10   a significant difference in the structure of the Lepi-
dopteran and Coleopteran toxin proteins.

Several approaches were taken to purifying
the individual protein components of the crystal.
Isoelectric focusing was not successful because all of
15   the protein precipitated. Anion exchange high pres-
sure liquid chromatograph (HPLC) on a Mono Q column
failed to resolve the components. Cation exchange
HPLC on a Mono S column in the presence of 4 M urea
resolved five peaks. Analysis of the peaks by SDS gel
20   electrophoresis indicated that peak A contained only
the higher molecular weight band from whole crystals.
Peak B was rich in this higher band with small amounts
of the lower band. Peak C was rich in the lower band
with significant amounts of the upper band. Peaks D
25   and E were mixtures of both bands. In most prepara-
tions the higher molecular weight band, corresponding
to peaks A and B, was the predominant protein in the
crystals. For the HPLC separated material, peaks A
and B represented most of the recovered protein.
30   The N-terminal amino acid sequences corres-
ponding to peaks A, B, and C were determined. Peaks A
and B were found to have the same N-terminal sequence
while the peak C sequence was different. The sequences
determined were:

Peak A and B:

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1               5                    10                   15
Met Asn Pro Asn Asn Arg Ser Glu His Asp Thr Ile Lys Thr Thr
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Peak C:

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1               5                    10                   15
Met  X  Pro  X  Thr Arg Ala Leu Asp Asp Thr Ile Lys Lys Asp

16
Val Ile Glyn Lys
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X represents an undeterminent amino acid.

10   Insect Toxicity of *B.t.t.* Proteins

Several preparations of *B.t.t.* and *B.t.t.*
proteins were tested for toxicity to various insects
including both Lepidopterans and Coleopterans. No
activity was observed towards Lepidopterans (corn
15   earworm, black cutworm, tobacco hornworm and cabbage
looper).   Among   the   Coleopterans,   activity   was
observed against Colorado potato beetle (*Leptinotarsa
decemlineata*) and boll weevil (*Anthonomus grandis*).
Lower level activity was exhibited against Southern
20   corn rootworm (*Diabrotica undecimpunctata howardi*).
Insecticidal activity was found in crude bacterial
cultures, purified crystals, solubilized crystals and
isolated peaks C, D, E (pooled), A and B.

Assays for toxicity to Colorado potato
25   beetle were carried out by applying the preparation to
be tested to tomato leaves and allowing the insects to
feed on the treated leaves for four days. Assays with
boll weevil and Southern corn rootworm were performed
by incorporating the test material in an appropriate
30   diet mixture.

IDENTIFICATION AND CLONING OF THE *B. t. t.*
TOXIN GENE IN *E. COLI* AND *PSEUDOMONAS*

Using this N-terminal protein sequence in-
formation, synthetic DNA probes (Figure 1) were
5   designed which were used in the isolation of clones
containing the *B. t. t.* toxin gene. Probes were end-
labeled with $[\gamma-^{32}P]$ ATP according to Maniatis (1982).
B. thuringiensis var. *tenebrionis* was grown for 6
hours at 37°C in Spizizen medium (Spizizen, 1958) sup-
10   plemented with 0.1% yeast extract and 0.1% glucose
(SPY) for isolation of total DNA. Total DNA was iso-
lated from *B. t. t.* by the method of Kronstad (1983).
Cells were grown on Luria agar plates for isolation of
B. t. t. crystals used in toxicity studies.

15   *E. coli* and *Pseudomonas* cultures were rou-
tinely grown in Luria Broth (LB) with ampicillin (Ap,
200µg/ml), kanamycin (Km, 50µg/ml), or gentamicin (Gm,
15µg/ml) added for plasmid selection and maintenance.

Isolation and Manipulation of DNA

20   Plasmid DNA was extracted from *E. coli and
Pseudomonas* cells by the method of Birnboim and Doly
(1979) and large quantities were purified using NACS-
52 resin (Bethesda Research Laboratories) according
to manufacturer's instructions. Restriction endonu-
25   cleases, calf alkaline phosphatase and T4 DNA ligase
were used according to manufacturer's instructions
(New England Biolabs). Restriction digestion products
were analyzed on 0.8% agarose gels electrophoresed in
Tris-acetate buffer. DNA fragments for cloning were
30   purified from agarose using the freeze-thaw method.
Construction of recombinant DNA molecules was accor-

ding to Maniatis et al. (1982). Transformation into
E. coli were performed according to Maniatis (1982).

Cloning of the *B.t.t.* Toxin Gene

5   Southern analysis (Southern, 1975) was
performed using the modified dried gel procedure
(Conner et al., 1983). Colony filter hybridization,
for detection of *B.t.t.* toxin clones, used the tetra-
methylammonium chloride method (Wood et al., 1985).

10   Southern analysis of BamHI and HindIII
digested *B.t.t.* total DNA identified a 5.8 kb BamHI
and a 3.0 kb HindIII fragment which hybridized to the
synthetic A1 probe. BamHI fragments of *B.t.t.* DNA
(5.4-6.5 kb) were purified from agarose gels and
ligated to alkaline phosphatase treated BamHI digested

15   pUC119. pUC119 is prepared by isolating the 476 bp
HgiAI/DraI fragment of bacteriophage M13 and making
the ends of the fragment blunt with T4 DNA polymerase
(New England Biolabs). This fragment is then inserted
into pUC19 that has been digested with NdeI and

20   filled with Klenow DNA polymerase (New England Bio-
labs). The ligated *B.t.t.* and pUC119 DNA was then
used to transform *E. coli* JM101 cells. After several
attempts only 150 Ap resistant colonies were obtained.
HindIII fragments of *B.t.t.* DNA (2.8-3.5 kb) were also

25   cloned into the HindIII site of pUC119, and 1100
colonies were obtained. All colonies were screened by
colony hybridization to the A1 probe (Figure 1). Ele-
ven HindIII clones showed strong hybridization, but
none of the BamHI colonies showed any hybridization.

30   The colonies identified by hybridization to A1 were
then screened using synthetic probe A2 (Figure 1) and
two colonies showed hybridization to the second probe.
Restriction digest patterns of the two colonies indi-

cated that the same 3.0 kb HindIII fragment was con-
tained in both but in opposite orientations. These
clones were designated pMON5420 and pMON5421 (Figure
3). To confirm that the clones did contain the gene
5   for the *B.t.t.* toxin protein, the single stranded DNA
from both clones was sequenced using degenerate probes
A1 and A2 as primers for di-deoxy sequencing (Sanger,
1977). Sequence analysis with A1 probe as primer
revealed an open reading frame (ORF) whose sequence
10 was identical to amino acids 9 through 15 of the amino
acid sequence determined for purified peaks A and B
of the *B.t.t.* toxin protein. Probe A2 produced DNA
sequence which began beyond the end of the determined
amino sequence, but this DNA sequence was identical
15 to sequence produced with A1. These results confirm
that the desired *B.t.t.* toxin gene was cloned.

Southern hybridization to total *B.t.t.* DNA
using degenerate probes based on the N-terminus of
peak C failed to detect specific bands suggesting
20 that the amino acid sequence determined for peak C
was incorrect or most probably was obtained from a
mixture of two or more proteins comprising peak C.

Analysis of Proteins Produced in *E. coli*

B.t.t. crystal proteins and recombinant
25 *B.t.t.* proteins were examined by SDS-PAGE (Laemmli,
1970). One ml of *E. coli* was centrifuged, the pellets
resuspended in 100µg SDS-sample buffer and 10µl
samples were electrophoresed on 7.5% polyacrylamide
gels. The gels were either stained with Coomassie
30 Blue or probed for cross reactivity to antibodies
raised against purified *B.t.t.* toxin crystals. Wes-
tern Blots were performed using the horseradish pero-
xidase conjugated antibody procedure (Towbin et al.,

1984). High molecular weight markers were purchased
from BioRad.

Further confirmation that the clones pro-
duced *B.t.t.* toxin was obtained by Western blot analy-
sis of the proteins produced in *E. coli*. *E. coli*
JM101 cells containing either pUC119, pMON5420 or
pMON5421 were grown overnight in the presence of IPTG
(0.1mM) to induce the lac promoter. Duplicate samples
were analyzed by SDS-PAGE along with purified *B.t.t.*
crystal proteins included as controls. Western blot
analysis of one gel revealed the production of 2 cross
reacting proteins by *E. coli* containing pMON5420 or
pMON5421. These proteins were identical in size to
the major and minor proteins of the *B.t.t.* crystal.
Molecular weights of the proteins were determined by
comparison to the molecular weight standards on the
second gel stained with Coomassie blue. The major
toxin protein was determined to be 74 kDa in size and
the minor toxin protein was determined to be 68 kDa in
size. The level of *B.t.t.* toxin proteins produced by
pMON5420 was increased by the addition of IPTG while
production of toxin proteins by pMON5421 was unaffected.

Production of *B.t.t.* Toxin(s) in
Pseudomonas fluorescens

A broad host range vector, pMON5432, was
constructed by cloning BamHI digested pMON5420 into
the BamHI site of pMON7111 as shown in Figure 2. This
vector was then mated into *P. fluorescens* 701E1 for
analysis of toxin production. Tri-parental matings
into *Pseudomonas fluorescens* were done as previously
described (Ditta et al., 1980). Samples of overnight
cultures, grown with and without IPTG, were prepared
for Western blot analysis and insect toxicity studies.

The proteins produced by *Pseudomonas* were identical in size to the *E. coli* produced proteins and protein expression was increased with the addition of IPTG.

Insect Toxicity Assay

Coleopteran toxin activity was assayed using newly hatched Colorado potato beetle (*Leptinotarsa decemlineata*) insects in a tomato leaf feeding assay. *E. coli* and *Pseudomonas* cultures were grown overnight in the presence of IPTG, centrifuged and resuspended at various concentrations in 10mM $MgSO_4$. The cells were disrupted by sonication (three 15 sec. pulsed treatments on ice). Tween-20 (0.1%) was added and the sample painted onto a tomato leaf placed into a 9cm petri dish lined with moist filter paper. Ten Colorado potato beetle larvae were added to each leaf. After four days, the percentage corrected mortality (percentage of insects alive in the control minus the percentage of insects alive in the treated sample divided by the percentage alive in the control) was computed using Abbott's formula (Abbott, 1925). Assays were performed in duplicate and the data combined. *B.t.t.* crystal/spore preparation were used as positive controls.

E. coli cultures of pMON5420 and pMON5421 were evaluated for Coleopteran toxicity using different concentrations of cultures grown with added IPTG. A comparison of recombinant and wild type *B.t.t.* toxin activities is shown below in Table I. The results show that the recombinant *B.t.t.* protein(s) are toxic to Colorado potato beetle. The 2x-concentrated, IPTG-induced pMON5420 culture killed 100% of the insects as did the *B.t.t.* spore/crystal control. These toxicity results demonstrate that the *B.t.t.*

gene cloned was the gene that encodes the *B.t.t.* toxin protein.

Insect feeding assay showed that the *Pseudomonas* produced toxins were toxic to Colorado potato beetle. The relative toxicity of *Pseudomonas* cultures was consistent with the amount of toxin protein produced as determined by Western blot analysis when compared to *E. coli* cultures.

TABLE I
Coleopteran Toxicity of Recombinant *B.t.t.* Toxin

Sample[1]	Concentration[2]	Corrected Mortality
E. coli JM101		
pUC119	2x	0%
pMON5420	1x	83%
pMON5420	2x	100%
pMON5421	1x	44%
pMON5421	2x	61%
P. fluorescens 701E1		
pMON5432	3x	60%
B.t.t. prep		100%

[1] Cultures were grown overnight with added IPTG, concentrated, sonicated and tested for toxicity.

[2] 1x equals cellular concentration of overnight culture.

SEQUENCE OF TOXIN GENE OF *B.t.t.*

Location and orientation of the *B.t.t.* gene within the cloned fragment was determined based· on the following information: a) DNA sequence was obtained from the single stranded pMON5421 template,

b) A PstI site identified, by DNA sequence analysis, near the start of translation was mapped in pMON5420 and pMON5421, c) several other restriction sites were mapped, d) a deletion from a BglII site to a BamHI

5   site which deletes 130 bp was constructed and both full-length proteins were produced. This information was used to construct maps of pMON5420 and pMON5421. Referring to Figure 4, the toxin coding region begins 500 bp from the 5' HindIII site, and 150 bp upstream

10   of the PstI site. The coding region ends approximately 450 bp from the 3' HindIII site. The BglII site is approximately 350 bp downstream of the stop codon.

Plasmids

15   The plasmids generated for sequencing the *B.t.t.* insecticidal toxin gene are listed in Table II. The parental plasmids, pMON5420 and pMON5421, are independent isolates of the HindIII fragment cloned into pUC119 in opposite orientation.

TABLE II

Sequencing Plasmids

pMON5420	3.0 HindIII insert from *B.t.t.* DNA (parent plasmid)
pMON5421	3.0 HindIII insert from *B.t.t.* DNA (parent plasmid)
pMON5307	EcoRI deletion of pMON5420
pMON5308	EcoRI deletion of pMON5421
pMON5309	PstI deletion of pMON5420
pMON5310	XbaI deletion of pMON5421
pMON5311	EcoRV-SmaI deletion of pMON5421
pMON5312	NdeI-BamHI deletion of pMON5421*
pMON5313	NdeI-BamHI deletion of pMON5420*
pMON5314	AsuII-BamHI deletion of pMON5421*
pMON5315	AsuII(partial)-BamHI deletion of pMON5421*
pMON5316	AsuII-BamHI deletion of pMON5421**
pMON5426	BglII-BamHI deletion of pMON5420
pMON5427	EcoRV-SmaI deletion of pMON5420
pMON5428	HpaI-SmaI deletion of pMON5420
pMON5429	XbaI deletion of pMON5420

* - After digestion of the DNA with both enzymes, the ends were filled in with Klenow polymerase, ligated and used to transform JM101.

** - Generation of the AsuII-BamHI deletion of this construct resulted in a rearrangement of an AsuII fragment to an orientation opposite to its original location. This resulted in a sequence of 5316 reading toward the NH$_2$ end.

Preparation of Single Stranded Template
for Sequencing

The following protocol provides reproducibly good yields of single stranded template for sequencing. A single colony containing the pUC119 with the fragment to be sequenced was streaked on L-agar (10 g tryptone, 5 g yeast extract, 5 g Nacl, and 15 g agar per liter) containing ampicillin (200μg per ml). A single colony from this plate was inoculated into 3 ml of L-broth (200μg per ml ampicillin) and incubated at 37°C overnight with shaking. From this culture, 50 μl was inoculated into 10 ml of 2X YT (20 g tryptone and 10 g yeast extract per liter) with 200 μg of ampicillin per ml in a 150 ml side arm flask and incubated at 37°C with shaking. After 2-3 hours (Klett reading of 50), 100 μl of M13K07 (helper phage) grown in *E. coli* JM101 was added to induce the culture. The flask was shaken for one hour followed by the addition of 20 ml of 2X YT adjusting the final concentration of kanamycin to 70μg per ml and ampicillin to 200 μg per ml. The cultures were shaken for 16-18 hours at 37°C. A total of three mls of the induced overnight culture was found to be sufficient to isolate a suitable amount of template for four sequencing experiments. The three mls were spun in 1.5 ml eppendorf tubes for 1 minute, decanted and filtered through a 0.2 um Gelman Sciences Acrodisc®. This step was found to be useful for the removal of cellular debris and intact *E. coli*. A polyethylene glycol precipitation (20% PEG, 2.5M NaCl, 500 μl per 2 ml of lysate) at room temperature for 10 minutes was followed by centrifugation for 10 minutes. The supernatant was discarded followed by a brief spin (15 seconds) and removal of the residual PEG. Any remaining PEG will be carried through the

template isolation and adversely affect DNA sequencing
reactions. The pellets are resuspended in 100 µl of
TE (10mM Tris, 1mM EDTA, pH 8.0), combined and mixed
well with 200 µl of buffered phenol (buffered by equi-
5   libration with an equal volume of 1M Tris-HCl, pH 8.0,
then 0.1M Tris-HCl, pH 8.0, followed by an equal vol-
ume of TE). After incubation at 55°C for 10 minutes
an equal volume (200 µl) of phenol/chloroform (1::1)
was added, vortexed, and centrifuged for 2 minutes.
10   The top layer was removed, extracted with 200 µl of
chloroform, centrifuged and the aqueous phase removed.
The single stranded template was precipitated with 25
µl of 3M sodium acetate (pH 5.2) and 600 µl of 95%
ethanol, incubated on dry ice for 5 minutes and cen-
15   trifuged for 10 minutes. The precipitate was resus-
pended in 25 µl of H_2O and 2 µl was checked on an
agarose gel for correct size, relative concentration
and contaminating DNA.

Sequencing Reagents and Conditions

20   The protocols for DNA sequencing are
described in detail in the Handbook available from
Amersham Corporation. Reagents (nucleotides, primer,
buffer, chase solution and Klenow polymerase) were
obtained from the Amersham M13 sequencing kit (catalog
25   #N4502). The sequencing mixes provided in the Amer-
sham kit were adjusted for efficient sequencing of the
A-T rich B.t.t. gene. Instead of the recommended 1::1
mix of dNTP to ddNTP, the following ratios were found
to be more appropriate; 40 µl dATP: 10 µl ddATP, 35 µl
30   dTTP: 15 µl ddTTP, 15 µl dGTP: 35 µl ddGTP, and 10 µl
dCTP: 40 µl ddCTP. Radioactive sulfur ($[\alpha-^{35}S]$ dATP)
was used in the sequencing reactions (Amersham catalog
#SJ.1304). The sequencing gels (prepared as described

in the Amersham handbook) were run on the Hoeffer
"Poker Face" apparatus at 70 watts (1200-1400 volts)
which was found to give very good resolution. Higher
voltages resulted in fuzzy bands.

5   Sequencing of the B.t.t. Toxin Gene

        The isolated plasmids, pMON5420 and pMON5421,
contained a 3.0 HindIII fragment in opposite orienta-
tion (see Figure 3). The major protein of the B.t.t.
crystal, which was used as the basis for design of the
10   oligonucleotide probes, has a molecular weight esti-
mated to be 73-76 kdal corresponding to approximately
2.0 kb of DNA. Initial sequencing from the A1 and A2
primers (synthetic oligonucleotides based on the amino
acid sequence of Peak A; see Table III, below) con-
15   firmed that the DNA sequence corresponded to the anti-
cipated amino acid sequence.

TABLE III
Synthetic Oligonucleotides Used for Sequencing
the B.t.t. Insecticidal Toxin Gene

Primer	Template	Sequence	Location[1]
Bttstart	pMON5420	tgaacatggttagttgg	291-275
Bttext	pMON5421	taggtgatctctaggcg	422-439
Bttseq	pMON5421	ggaacaaccttctctaatat	1156-1175
BttA1*	pMON5421	atgaayccnaayaaycg	205-222
BttA2*	pMON5421	garcaygayacyathaa	227-242

* y = t or c.   r = a or g. h = t,c or a. n = a,g,c or t.

[1] The location of the primers is based on the total of 2615
bases sequenced. Sequencing from pMON5420 proceeded
toward the amino acid end and from pMON5421 toward the
carboxyl end (see Figure 3).

A PstI site was located in the initial sequence which was used to identify the location and probable orientation of the *B.t.t.* gene within pMON5420 and pMON5421 (see Figures 3 and 4). Mapping of restriction sites with a number of enzymes (HpaI, XbaI, NdeI, EcoRV, and BglII) and the numerous unique sites remaining in the pUC119 portion of both pMON5420 and pMON5421 provided the opportunity to obtain sequence using the universal sequencing primer. Deletions were generated in both pMON5420 and pMON5421 bringing the universal primer homologous region in close proximity to internal regions of the gene. In areas not easily sequenced by generating deletions, synthetic oligonucleotides corresponding to sequenced regions in the coding sequence (Table III) were used as primers to obtain extensions of the sequenced regions. The regions sequenced (sequence coordinates; Table IV) and the direction of sequencing is depicted in Figure 4.

TABLE IV
Source of Sequence Data

Plasmid	Length (bp)	Location	Plasmid	Length (bp)	Location
pMON5307	414	797-1211	pMON5316	153	1861-2041
pMON5308	276	1895-2171	pMON5426	300	2220-2520
pMON5309	170	114-284	pMON5427	110	1701-1812
pMON5310	283	1595-1880	pMON5428	129	1548-1677
pMON5311	110	1812-1922	pMON5429	303	1292-1595
pMON5312	248	782-1030	Bttstart	264	1-264
pMON5314	291	2041-2305	Bttext	380	440-820
pMON5315	330	1157-1187	BttA2	267	250-517

COMPUTER ANALYSIS OF THE *B.t.t.*
INSECTICIDAL TOXIN GENE

A total of 2615 base pairs of sequence were obtained from pMON5420 and pMON5421. Computer analysis of the sequence revealed a single open reading frame from base pair 205 to 2136. Referring to Figure 5, the *B.t.t.* insecticidal toxin gene is 1932 base pairs, coding for protein of 644 amino acids with a molecular weight of 73,091 daltons. The protein has a net charge of -17 and a G-C content of 34%.

Comparison Between Coleopteran-type and
Lepidopteran-type Toxin Genes and Proteins

Although the Coleopteran-type toxins and the Lepidopteran-type toxins are derived from *Bacillus thuringiensis*, there are significant differences between the toxin genes and the toxin proteins of the two types. As isolated from *Bacillus thuringiensis* both types of toxins are found in parasporal crystals; however, as described above, the solubility properties of the crystals are distinctly different. In addition, the sizes of the toxin proteins found in solubilized crystals are completely different. Lepidopteran-type toxin proteins are typically on the order of 130 kDa while the Coleopteran-type toxin proteins are approximately 70 kDa.

Isolation and DNA sequence analysis of the Coleopteran-type toxin gene from *B.t. tenebrionis* predicts the amino acid sequence of the toxin protein (see Figure 5). Both the nucleotide sequence and the derived amino acid sequence of the Coleopteran-type toxin gene have been compared to nucleotide and amino acid sequence of a typical Lepidopteran-type toxin.

This comparison was performed using the computer program BESTFIT of Devereux et al (1984) which employs the algorithm of Smith and Waterman (1981). BESTFIT obtains maximum alignment of two nucleotide or amino acid sequences. BESTFIT calculates two parameters, quality and ratio, which can be used as alignment metrics when comparing different alignments. Ratio varies between 0 and 1.0. A larger ratio indicates a better alignment (greater similarity) between two sequences.

The BESTFIT alignment shows that the two types of toxin genes are related at both the nucleotide sequence and amino acid sequence level. However, the alignment also shows that the two sequences are clearly distinct and possess many regions of mismatch at both the nucleotide and amino acid sequence levels. For example, the ratio for comparison of the two amino acid sequences is only 0.22. At the nucleotide sequence level, maximum alignment is obtained only by the introduction of many gaps in both sequences, and the ratio is only 0.072.

There are many sequenced examples of Leptidopteran-type toxin genes; similar comparison among these genes has shown that the gene from *B. t. kurstaki HD-1* described by Schnepf et al. (1985) and that from *B. t. kurstaki HD-73* described by Adang et al. (1985) represent the two most divergent Lepidopteran-type toxin genes. By comparison with the ratios calculated above for alignment of the Colepteran-type and the Lepidopteran-type gene, the ratio for amino acid sequence comparison of the two most divergent Lepidopteran-type proteins is 0.811, and the ratio for these two Lepidopteran-type genes at the nucleotide sequence level is 0.755. This indicates that although the

Coleopteran-type and Lepidopteran-type toxin genes may be evolutionarily related, they are quite distinct in both nucleotide and amino acid sequence.

HIGH LEVEL PRODUCTION OF RECOMBINANT
B. t. t. TOXIN IN E. COLI

To facilitate purification of large quantities of recombinant B. t. t. toxin, it was necessary to clone the B. t. t. gene into an E. coli high expression vectors. Site directed mutagenesis was used to introduce an NcoI restriction site into pMON5420 at the ATG codon at the start of the open reading frame.

Site Directed Mutagenesis

Site-directed mutagenesis to introduce new restriction sites was performed by the method of Kunkel (1985). Plasmid pMON5420 was introduced by transformation into E. coli strain BW313, which contains the dut^- and ung^- mutations in order to incorporate deoxyuridine into the DNA. A single transformed colony was grown overnight in 2X YT medium containing 100 µg/ml ampicillin and 0.25 µg/ml uridine. A 0.5 ml aliquot of this culture was added to 10 ml of the same medium and incubated for one hour at 37°C with vigorous shaking to a density of 0.23 (A600). To induce formation of single strand containing phage particles, helper phage M13K07 was added at a multiplicity of approximately 10 and incubation was continued for one hour to a density of 0.4 (A600). The culture was diluted by addition of 30 ml of the above medium, and kanamycin was added to a final concentration of 70 µg/ml. Incubation was continued for 15 hours at which point cells were removed by centrifuga-

tion. Phage particles were precipitated from 25 ml of supernatant by addition of 5 ml of 20% PEG/2.5 M NaCl/50 µg/ml RNAase A followed by incubation on ice for 15 minutes. Phage were recovered by centrifuga-

5 tion and dissolved in 0.8 ml TE buffer. DNA was isolated from the particles by three extractions with 0.8 ml phenol/chloroform/isoamyl alcohol (25:24:1) followed by ethanol precipitation. The DNA pellet was dissolved in 100 µl of water to a final concentration

10 of approximately 1 mg/ml (estimated by agarose gel electrophoresis).

Synthetic oligonucleotide primers for muta-genesis were suspended in water at a concentration of approximately 10 pmole/µl. The oligonucleotides

15 were phosphorylated utilizing T4 polynucleotide kinase in a reaction containing 50 pmoles oligonucleotide, 1 mM ATP, 25 mM Tris-Cl pH 8, 10 mM $MgCl_2$, 0.2 mM spermidine-HCl, 1 mM DTT and 2 units of enzyme. The reaction was incubated at 37°C for 30 minutes and then

20 heated at 70°C for 5 minutes. The phosphorylated primer was annealed to the deoxyuridine containing phage DNA by mixing approximately 1 pmole of the phage DNA (2 µg) with 10 pmole primer in a reaction con-taining 6.6 mM Tris-HCl, 6.6 mM $MgCl_2$, 6.6 mM NaCl and

25 5 mM DTT. The mixture was heated to 70°C for seven minutes and then slowly cooled to room temperature. The annealed primer/template was used as the substrate for synthesis of double-stranded, closed circular DNA by addition of each dNTP to 0.5 mM, ATP to 0.5 mM, 5

30 units of Klenow fragment DNA polymerase and 400 units T4 DNA ligase (New England Biolabs). The reaction was carried out in the same buffer salts as for annealing at 15°C for approximately 15 hours. At this time an additional 400 units of ligase was added and incuba-

35 tion was continued for two hours.

One half of the reaction was used to trans-
form 0.15 ml of CaCl$_2$-treated JM101 cells, and the
cells were spread on LB plates containing 100 µg/ml
ampicillin. Between 30 and several hundred colonies
5   were recovered for each mutagenesis reaction. Single
colonies were grown overnight in LB containing ampi-
cillin and plasmid minipreps were prepared by the
alkaline SDS method. Plasmids were analyzed for the
presence of the new restriction site and the presence
10   of the site was confirmed by sequence analysis as
described above.

A plasmid containing a NcoI site (pMON9759)
at the start of the B.t.t. insecticidal toxin gene was
generated by site-specific mutagenesis. The primer
15   used is shown below:

Desired Site   Primer

   NcoI   GATTGTTCGGATCCATGGTTCTTCCTCCCT

The generation of the NcoI site at the N-terminus has
20   changed the second amino acid from asparagine to
aspartic acid. This change does not affect insect
toxicity. BamHI and StyI sites have also been gener-
ated as a consequence of the introduction of this NcoI
site. The plasmid containing the NcoI site has been
25   designated pMON9759. The 2.5 kb NcoI-HindIII fragment
containing the toxin encoding segment from pMON9759
was then cloned into NcoI-HindIII digested pMON5634 to
produce pMON5436. Referring to Figure 16, pMON5634
is a pBR327 based plasmid which also contains the fl
30   phage origin of replication. The vector contains a
synthetic recA promoter which is induced by nalidixic
acid. The gene 10 leader from phage T7 (described in

commonly assigned U.S. patent application serial
number 005821, filed February 4, 1987, the disclosure
of which is hereby incorporated by reference) is also
present to increase expression in *E. coli.* A synthe-
5   tic linker with multiple cloning sites was added for
insertion of genes downstream of the promoter and gene
10 leader sequence.

For induction of the *recA* promoter, over-
night cultures were diluted 1:50 into M9 minimal media
10   (Miller, 1972) with 0.2% casamino acids and 0.25% glu-
cose added. At 150 Klett units, naladixic acid was
added to 50µg/ml and cells were harvested 3 hours post
induction. The level of *B.t.t.* toxin produced by
nalidixic acid induced pMON5436 was compared to IPTG
15   induced pMON5420 by analysis on SDS-PAGE. The Coomas-
sie blue stained gel revealed no detectable *B.t.t.*
produced by pMON5420 while the level of *B.t.t.* pro-
duced by pMON5436 was approximately 5% of total
protein. This construct was used to isolate large
20   quantities of the recombinant *B.t.t.* toxin proteins
to investigate toxicity levels, insect specificity,
and mode of action.

B.t.t. TOXIN CHARACTERIZATION

Identification of the Number and Origin
25   of the *B.t.t.* Proteins

B.t. var. *tenebrionis* produces a number of
Coleopteran-type toxin proteins, present in protein
crystals, which are produced co-incidentally with spo-
rulation (see Figure 6). These protein crystals are
30   released into the media as cells autolyse during or
following sporulation. To determine the number of
toxin proteins produced by *B.t.* var. *tenebrionis*, 500

ml cultures of this organism were grown in 2 liter flasks in 15% TSB medium in 100 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer, pH 7.0 at 30°C for 7 days. At this point the cultures have sporulated and the cells lysed. Protein crystals and spores were harvested by centrifugation at 20,000 x gravity (g) for 20 min. at 4°C. Pellets were washed three times with excess water, followed by three washes with 2 M NaCl. The resultant pellet was stored at 4°C in water plus 0.02% sodium azide. *B.t.t.* toxin protein was solubilized from the crystals by suspending the pellet in 100 mM sodium carbonate buffer, pH 10 and stirring this suspension for two hours at room temperature. After centrifugation 20,000 x g for 20 min to remove unsolubilized materials, the supernatant was filtered through a 0.2 μm filter to remove any remaining spores. *B.t.t.* toxin protein prepared in this manner, as do crystals solubilized in 125 mM Tris-HCl, 4% SDS, 20% glycerol and 10% 2-mercaptoethanol, pH 6.8, (SDS sample buffer used to prepare samples for SDS-PAGE analysis) is comprised of four major and different proteins as judged by SDS-PAGE analysis. Five unique products were identified by N-terminal amino acid analysis. To determine whether all five of these proteins were derived from the same gene or whether two or more genes are required for their synthesis, the N-terminal amino acid sequence of each of these proteins were determined using automatic Edman degradation chemistry.

An Applied Biosystems, Inc. Model 470A gas phase sequencer (Foster City, CA) was employed (Hunkapiller, et al., 1983). The respective PTH-amino acid derivatives were identified by RP-HPLC analysis in an on-line fashion employing an Applied Biosystems, Inc.

Model 120A PTH analysis fitted with a Brownlee 2.1 mm
I.D. PTH-C18 column. Determination of the N-terminal
amino acid sequence of each protein will establish
whether all these proteins were derived from the
5   B.t.t. toxin gene described above.

The strategy to sequence these proteins was
to sequence the B.t.t. toxin proteins corresponding to
bands 1 and 3 (see Figure 6) from the E. coli clone
JM101 (pMON5436), bands 2, 3 and 4 by electro-elution
10   of the proteins produced by B.t. var. tenebrionis
from SDS-PAGE gels. The sequence of B.t.t. 1 and 3
was determined with proteins purified from JM101
(pMON5436). JM101 (pMON5436), as well as the other
E. coli constructs (pMON5450, 5456 and 5460, infra)
15   produces the B.t.t. in the form of insoluble refrac-
tile bodies after cultures are induced for high level
expression. The E. coli constructs were grown in
modified M9 media at 37°C. A culture grown overnight
was used to inoculate 400 ml of the modified M9 media
20   in 2.4 1 fernbach flasks to an initial starting den-
sity of 10 Klett units. Nalidixic acid, in 0.1 N
NaOH, was added to the cultures at 100 Klett units to
a final concentration of 50 µg/ml, to induce B.t.t.
toxin protein expression. After an additional 4 hours
25   of incubation, cultures were harvested by centrifuga-
tion at 20,000 x g for 20 min. at 4°C. Cell pellets
were suspended in water to a density equivalent to
5000 Klett units per ml and sonicated in an ice bath
with a Heat Systems Ultrasonics sonicator at a power
30   of 9, 50% duty cycle for a total of 5 min. The soni-
cated preparation was centrifuged for 20 min. at
20,000 x g at 4°C. Pellets, containing refractile
bodies and cell debris, were washed twice with cold
water and suspended at 10,000 Klett unit equivalents

per ml in water plus 25% sulfolane. After stirring at room temperature for 2 hours, the solubilized refractile body preparations were centrifuged again at 20,000 x g at 4°C to remove unsolubilized materials. Tris-HCl was added to the supernatant to a final concentration of 50 mM, pH 7.6. The *B.t.t.* bands 1 and 3 were co-purified on an HR5/5 MonoQ ion exchange column using a 75 to 200 mM Nacl gradient in 50 mM Tris-HCl, 25% sulfolane, pH 7.6. Fractions containing *B.t.t.* bands 1 and 3 were identified by 9% SDS-PAGE analysis, pooled, dialyzed into 100 mM sodium carbonate, pH 10 buffer and concentrated in Amicon centricon concentrators. *B.t.t.* toxin protein corresponding to band 3 was purified from JM101 (pMON5456) in an analogous manner.

Bands corresponding to 2 alone and bands 3,3' and 4 (see Figure 6) combined were electro-eluted from 7% SDS-PAGE slab gels which were run with 48 µg of *B.t.t.* crystals solubilized in 100 mM sodium carbonate, 20 mM dithiotheitol (DTT), pH 10 buffer. Gels were stained for 10 min in Coomassie blue R250 and destained in 50% methanol, 10% acidic acid for 20 min. Appropriate bands were excised with a razor blade and the *B.t.t.* protein electro-eluted. Knowing the amino acid sequence, deduced from the DNA sequence of the *B.t.t.* toxin gene cloned in *E. coli*, all five N-termini of these unique proteins were identified (Figure 7).

Proteins corresponding to band 1 and 3 originated from two independent translational initiation events which start at the methionine at positions 1 and 48 (Figures 6 and 7), respectively. Proteins corresponding to *B.t.t.* bands 2, 3 and 4, observed only in *B.t.* var. *tenebrionis* and not in the *E. coli*

constructs, apparently arise from proteolytic cleavage of either bands 1 or 3. These results establish that all five proteins originate from the same gene.

Purification of _B.t.t._ Bands 1 and 3 for
Insect Toxicity Testing

The _B.t.t._ proteins produced in _E. coli_ corresponding to bands 3 and 1 plus 3 which were solubilized in 25% sulfolane and purified by MonoQ chromatography for N-terminal amino acid sequence analysis showed no insect toxicity against Colorado potato beetle insects. In subsequent experiments, it was demonstrated that sulfolane itself inactivates _B.t.t._ Therefore, an alternative purification method was developed and used compare the relative insecticidal toxicities of _B.t.t._ bands 1 and 3 produced in _E. coli_ compared to the _B.t.t._ solubilized from native crystals of _B.t._ var. _tenebrionis_. Cultures were grown, induced, harvested and refractile bodies isolated as described above. The various _B.t.t._ proteins were solubilized from the refractile bodies using 100 mM sodium carbonate, pH 10. The solubilized _B.t.t._ toxin, concentrated using Amicon stirred cells with YM-10 membranes, was purified on a Pharmacia Superose-12, gel filtration FPLC column, which separates _B.t.t._ bands 1 and 3 and from other contaminating proteins. Appropriate fractions, based upon SDS-PAGE analysis, were pooled, concentrated and used for insect toxicity experiments with the Colorado potato beetle insects. Proteins corresponding to band 1 (pMON5436, band 1 (pMON5460) and band 3 (pMON5456) were greater than 90% pure based upon SDS-PAGE analysis. Band 1 produced by pMON5460 has isoleucine at amino acid 48 in place of methionine (see below).

To obtain native protein toxin from *B.t.* var. *tenebrionis* for toxicity comparisons, native crystals were isolated and purified using sucrose gradient centrifugation as described above. Crystals were solubilized in 100 mM sodium carbonate, 20 mM DTT, pH 10 and used for insect toxicity tests.

All *B.t.t.* toxin protein preparations and controls for insect assay contained 0.3% Tween 20, a surfactant which enhances the ability of these solutions to bind to tomato leaves. Insect toxicity experiments were performed by thoroughly painting the upper and lower surfaces of 3 to 4 week old detached tomato leaves with buffer solutions containing the designated *B.t.t.* proteins at the indicated protein concentrations. After the solutions were air dried on the surface of the tomato leaves, a single leaf and 10 Colorado potato beetle insects were placed in a petri dish and incubated at 22°C for 4 days. The number of dead insects was determined and the toxicity results expressed as % corrected mortality (%CM); according to Abbott's formula described above. All experiments were performed in duplicate and all but the *B.t.t.* band 1 from pMON5460 were repeated on different days. The results of these tests are shown in the table below.

TABLE V

Toxicity of *B. t. t.* Proteins
Against Colorado Potato Beetle

Sample	Concentration (ug/ml)	Corrected Mortality (%)
B. t. t. Solubilized	100	100
	20	70
	4	10
Purified Band 1 (pMON5436)	100	87
	20	68
	10	34
Purified Band 1 (pMON5460)	100	67
	20	72
	10	44
Purified Band 3 (pMON5456)	100	91
	20	64
	10	32

Relative toxicity of purified proteins from different *E. coli* constructs were compared to solubilized native *B. t. t.* crystals. Band 1 (pMON5436) and Band 3 (pMON5456) were purified as described. Band 1 (pMON5460) was purified using gel filtration chromato-graphy. Native *B. t. t.* crystals were solubilized in 100 mM Na_2CO_3, pH 10.

The amounts of *B. t. t.* toxin required to kill 50% of the Colorado potato beetle insects were essen-tially identical for *B. t. t.* band 1 isolated from pMON5436 and pMON5460 and *B. t. t.* band 3 isolated from pMON5456 (Table V). Likewise, all of these purified *B. t. t.* preparations from *E. coli* demonstrated toxici-ties essentially identical to that observed with the sodium carbonate solubilized native toxin from *B. t.* var. *tenebrionis.*

DETERMINATION OF TOXIC FRAGMENTS
OF *B.t.t.* TOXIN PROTEINS

Several groups (Schnepf et al. 1985, Hofte
et al. 1986, and Wabiko et al. 1986) have reported
that C-terminal truncations of the Lepidopteran-type
toxins do not reduce toxicity (of the 1155 amino acids
a truncation to amino acid 607 did not result in a
loss of toxicity). Therefore, the C-terminal half of
the protein is not required for toxicity. Others have
also reported that the Lepidopteran-type toxin genes
which contain C-terminal deletions are more highly
expressed in transformed plants. There are also
reports that to retain toxicity, only small trunca-
tions can be made at the N-terminus (Schnepf et al.
1985, and Hofte et al. 1986). Contrary to those
teachings it has now been found that the Coleopteran-
type toxin of *B.t.t.* has substantially different pro-
perties. That is, the C-terminal portion appears to
be critical for toxicity therefore permitting essen-
tially no truncations. However, N-terminal deletions
can be made and maintain toxicity. These differences
were uncovered using the constructs described below:

Construction of pMON5426 (BglII/BamHI Deletion)

pMON5420 was digested with BglII and BamHI,
ligated and transformed into JM101 to create pMON5426.
This deletion was constructed to confirm that the
BglII site was not within the coding region of the
B.t.t. toxin gene.

Construction of pMON5438 (HpaI, C-terminal
Deletion of 463 bp)

pMON5420 was digested with HpaI and ligated with the following synthetic terminator linker. The linker contains nonsense codons in each reading frame and a BglII 5' overhang.

5'-TAGTAGGTAGCTAGCCA-3'

3'-ATCATCCATCGATCGGTCTAG-5'

The ligation was digested with BglII, to remove multiple linker inserts and then re-ligated. The ligation was transformed into JM101 and pMON5430 was isolated. To generate a NcoI site at the start of the truncated gene, the 2.32 kb PstI fragment of pMON9759 was replaced with the 1.47 kb PstI fragment of pMON5430 and the new construct was designated pMON5434. The 1.57 kb NcoI/HindIII fragment from pMON5434 was cloned into the *E. coli* high expression vector pMON5634, to create pMON5438.

Construction of pMON5441 (EcoRV, C-terminal
Deletion of 327 bp)

pMON5420 was digested with EcoRV and ligated with the synthetic terminator linker. The ligation was digested with BglII, to remove multiple linker inserts and then re-ligated. The ligation was transformed in JM101 and pMON5431 was isolated. To generate a NcoI site at the start of the truncated gene, the 2.32 kb PstI fragment of pMON9759 was replaced with the 1.61 kb Pst fragment of pMON5431, and the new construct was designated pMON5435. The 1.71 kb NcoI/HindIII fragment from pMON5435 was cloned into the *E. coli* high expression vector pMON5433 to create pMON5441.

Construction of pMON5449 (Bal31, C-terminal Deletion of 190 bp)

BglII digested pMON9759 was treated with Bal31 nuclease for 5 min. following the manufacturer's instructions. The DNA was electrophoresed in a 0.8% agarose gel and purified from the agarose by the freeze thaw method. The synthetic terminator linker was then ligated to the purified DNA and pMON5442 was isolated. The NcoI/BglII fragment of pMON9759 was replaced with the truncated gene fragment from pMON5442 to create pMON5445. The NcoI/HindIII fragment from pMON5445 was cloned into the *E. coli* high expression vector pMON5634 to create pMON5449. The endpoint at the Bal31 created deletion was determined by DNA sequence analysis.

Construction of pMON5448 (XmnI, C-terminal Deletion of 16 bp)

pMON5436 was digested with XmnI and ligated with the synthetic terminator linker. The ligation was then digested with NcoI and BglII and the 1.92 kb NcoI/BglII fragment containing the truncated gene was cloned into NcoI and BlgII digested pMON9759 to replace the full-length gene and create pMON5446. The NcoI/HindIII fragment from pMON5446 was cloned into *E. coli* high expression vector pMON5634 to create pMON5448.

Construction of pMON5450 (NcoI fill-ends, Removal of First ATG from Toxin ORF

pMON5436 was digested with NcoI, the ends filled using Klenow fragment DNA polymerase, ligated and transformed into JM101 to create pMON5450. This plasmid expresses only band 3 protein.

Construction of pMON5452 (N-terminal, Deletion of 224 bp)

The *B.t.t.* gene contains two StyI sites (227 and 1587) and a third site was added by the mutagenesis to create a NcoI site in pMON9759. The following experiments were performed to delete 5' *B.t.t.* DNA to base pair 227. pMON5434 (HpaI deletion derivative described above) was digested with StyI, the ends filled with Klenow DNA polymerase, ligated, and transformed into JM101 to isolate pMON5444. This manipulation destroys both the NcoI and StyI cleavage sites. This manipulation creates an in frame fusion with the first methionine (amino acid 1) and leucine (amino acid 77). The C-terminus of the gene was added by cloning the 1.9 kb NdeI/KpnI fragment from pMON9759 into pMON5444 to create pMON5452.

Construction of pMON5456 (Band 3 Mutant, N-terminal Deletion of 140 bp)

A NcoI site was introduced into pMON5420 at the ATG for band 3 by site directed mutagenesis as described above using the primer:

Mutagenesis Primer - BTTLOOP

CGTATTATTATCTGCATCCATGGTTCTTCCTCCCT

to create pMON5455. The mutagenesis also deleted the upstream sequence which encodes the N-terminal 48 amino acids of band 1. The NcoI/HindIII fragment from pMON5455 was cloned into the *E. coli* high expression vector pMON5634 to create pMON5456. This plasmid expresses only band 3. The generation of the NcoI site changes the second amino acid from thionine to aspartic acid.

Construction of pMON5460 (Mutant Band 1 Gene with MET48 Changed to ILE)

The codon for methionine at position 48 in pMON9759 was changed to a codon for isoleucine by site directed mutagenesis as described above using the primer:

Mutagenesis Primer - BTTMET
ATTATTATCTGCAGTTATTCTTAAAAACTCTTTAT

to create pMON5458. The NcoI/HindIII fragment of pMON5458 was cloned into the *E. coli* high expression vector pMON5634 to create pMON5460. By removing the ATG codon which initiates translation of band 3 protein, pMON5460 produces only band 1 protein with an isoleucine residue at position 48.

Construction of pMON5467 (Band 5 Mutant, N-terminal Deletion of 293 bp)

A NcoI site was introduced into pMON5420 to create a N-terminal deletion of ninety-eight amino acids by site directed mutagenesis using the primer:

Mutagenesis Primer
TCACTTGGCCAAATTGCCATGGTATTTAAAAAGTTTGT

to create pMON5466. A methionine and alanine were also inserted by the mutagenesis. The NcoI/HindIII fragment from pMON5466 was cloned into the *E. coli* high expression vector pMON5634 to create pMON5467.

INSECT TOXICITY RESULTS

C-Terminal Truncations

Coleopteran-toxin activity was determined using newly hatched Colorado potato beetles in a tomato leaf feeding assay as previously described. The mutant *B. t. t.* genes used for analysis of the C-terminus are shown in Figures 8 and 10. pMON5438 contains 490 amino acids of *B. t. t.* toxin protein plus 3 amino acids encoded by the linker used in the vector construction. The truncated protein was produced at high levels in *E. coli,* but had no activity against Colorado potato beetle. pMON5441 produces a protein which contains 536 amino acids of the *B. t. t.* toxin. The truncated protein was produced at high levels in *E. coli* but had no activity against Colorado potato beetle. pMON5449 contains 582 amino acids of the *B. t. t.* protein plus two amino acids encoded by the linker used in the vector construction. The truncated protein was produced at high levels in *E. coli,* but had no activity against Colorado potato beetle. pMON5448 contains 640 amino acids of the *B. t. t.* protein plus 2 amino acids encoded by the linker used in the vector construction. The truncated protein was produced at high levels by *E. coli,* but the protein had no activity against Colorado potato beetle. These results suggest that the C-terminus of the *B. t. t.* toxin protein is required for toxicity to Colorado potato beetle. A deletion of only 4 amino (pMON5448) acids resulted in a complete loss of activity. These results are directly contrary to the reported litera-ture with respect to Lepidopteran-type *B. t.* toxins.

Results for N-Terminal Mutations and Deletions

         The other mutant *B.t.t.* genes used for ana-
lysis of the N-terminus are shown in Figures 9 and 10.
Analysis of protein produced by pMON5450 revealed that

5   band 3 production in *E. coli* was due to translation
initiation at MET48 rather than a product of protease
cleavage. Toxicity studies also showed that band 3
was toxic. pMON5456 produces a protein which begins
at amino acid 48 with amino acid 49 changed from

10 threonine to aspartic acid. This protein was pro-
duced at high levels in *E. coli* and was toxic to
Colorado potato beetle. pMON5452 produces a protein
which begins at amino acid 77. This protein was
expressed in *E. coli*, and it had activity against

15 Colorado potato beetle. pMON5467 produces a protein
which begins at amino acid 99 and has two amino acids
added to the N-terminus (methionine and alanine).
This protein was produced in *E. coli* and exhibited no
detectable activity against Colorado potato beetle,

20 however, the level of expression for this deletion
variant was significantly lower than other variants.
These results suggest that the N-terminus of the *B.t.t.*
toxin protein can tolerate deletions. A deletion of
76 amino acids exhibitied toxicity. A deletion of 99

25 amino acids did, however, result in a loss of toxicity.
pMON5460 contains a mutation which changed methionine
at position 48 to isoleucine to prevent production of
band 3. The toxicity of band 1 produced by pMON5460
was equal to the toxicity of band 3 produced by

30 pMON5456.

CONSTRUCTION OF PLANT TRANSFORMATION VECTORS

The *B.t.* var. *tenebrionis* toxin gene con-
tained in pMON5420 was modified for incorporation into
plant expression vectors. A BglII site was introduced
5   just upstream of the ATG codon which specifies the
initiation of translation of the full-length *B.t.t.*
toxin protein (referred to as band 1) using the site
specific mutagenesis protocol of Kunkel (1985) as pre-
viously described. The sequence of the *B.t.t.* toxin
10   gene in the region of the initiator ATG is:

ATGATAAGAAAGGGAGGAAGAAAAATGAATCCGAACAATCGAAGTGAACATGATACAATA
                        MetAsnProAsnAsnArgSerGluHisAspThrIle

The primer for this mutagenesis (bttbgl) was 27 nucleotides in
length and has the sequence:

15   CGGATTCATT TTAGATCTTC CTCCCTT

Following mutagenesis a plasmid containing
the new BglII site was identified by digestion with
BglII and the change was verified by DNA sequence
analysis. The resulting plasmid containing the *B.t.t.*
20   toxin gene with the new BglII site was designated
pMON9758 (Figure 11).

The *B.t.t.* toxin gene in pMON9758 was
inserted into the expression cassette vector pMON316
(Sanders et al., 1987). pMON316 contains the CaMV35S
25   promoter and the 3' end from the nopaline synthase
(NOS) gene with a BglII site for gene insertion
between these two elements. Plasmid pMON9758 was
digested with BglII and a fragment of approximately
2.3 kb was isolated. This fragment extends from the

BglII site just upstream of the ATG codon to a BglII
site found approximately 350 bp downstream of the ter-
mination codon for the *B.t.t.* toxin gene. Thus, this
fragment contains the complete coding sequence of
the *B.t.t.* gene and also about 350 bp of noncoding
sequence 3' to the termination codon. This BglII
fragment was ligated with BglII digested pMON316.
Following transformation into *E. coli*, a colony was
identified in which the *B.t.t.* toxin gene was inserted
into pMON316 such that the 5' end of the toxin gene
was adjacent to the CaMV35S promoter. This plasmid
was designated pMON9753. A plasmid containing the
B.t.t. toxin gene in the opposite orientation in
pMON316 was isolated and designated pMON9754 (Figure
11).

Both pMON9753 and pMON9754 were introduced
by a triparental mating procedure into the *Agrobac-
terium tumefaciens* strain ASE which contains a dis-
armed Ti plasmid. Cointegrates between pMON9753 or
pMON9754 and the disarmed Ti plasmid were identified
as described by Fraley et al. (1985), and their
structures confirmed by Southern analysis of total
Agrobacterium DNA.

Additional plant expression vectors con-
taining the *B.t.t.* toxin gene have also been con-
structed (see Figures 12 and 13). In these vectors
the *B.t.t.* toxin gene has been inserted into the plant
expression vector pMON893 (Figure 14). Referring to
Figure 14, the expression cassette pMON893 consists of
the enhanced CaMV35S promoter and the 3' end including
polyadenylation signals from a soybean gene encoding
the alpha-prime subunit of beta-conglycinin (referred
to below as the "7S gene"). Between these two ele-

ments is a multi-linker containing multiple restriction sites for the insertion of genes.

The enhanced CaMV35S promoter was constructed as follows. A fragment of the CaMV35S promoter extending between position -343 and +9 was previously constructed in pUC13 by Odell et al. (1985). This segment contains a region identified by Odell et al. (1985) as being necessary for maximal expression of the CaMV35S promoter. It was excised as a ClaI-HindIII fragment, made blunt ended with DNA polymerase I (Klenow fragment) and inserted into the HincII site of pUC18. The upstream region of the 35S promoter was excised from this plasmid as a HindIII-EcoRV fragment (extending from -343 to -90) and inserted into the same plasmid between the HindIII and PstI sites. The enhanced CaMV35S promoter thus contains a duplication of sequences between -343 and -90 (see Figure 18).

The 3' end of the 7S gene is derived from the 7S gene contained on the clone designated 17.1 (Schuler et al., 1982). This 3' end fragment, which includes the polyadenylation signals, extends from an AvaII site located about 30 bp upstream of the termination codon for the beta-conglycinin gene in clone 17.1 to an EcoRI site located about 450 bp downstream of this termination codon.

The remainder of pMON893 contains a segment of pBR322 which provides an origin of replication in E. coli and a region for homologous recombination with the disarmed T-DNA in Agrobacterium strain ACO (described below); the oriV region from the broad host range plasmid RK2; the streptomycin resistance/sprectinomycin resistance gene from Tn7; and a chimeric NPTII gene, containing the CaMV35S promoter and the nopaline synthase (NOS) 3' end, which provides kanamycin resistance in transformed plant cells.

pMON9753 contained approximately 400 bp of 3' noncoding sequence beyond the termination codon. Since this region is not necessary for toxin production it was removed from the *B.t.t.* toxin gene segments inserted in pMON893. In order to create a *B.t.t.* toxin gene containing no 3' flanking sequence, a BglII site was introduced just after the termination codon by the method of Kunkel (1985). The sequence of the *B.t.t.* toxin gene around the termination codon is:

GTTTATATAGACAAAATTGAATTTATTCCAGTGAATTAAATTAACTAGAAAGTAAAGAAG
ValTyrIleAspLysIleGluPheIleProValAsnEnd

Mutagenesis was performed with a primer (bttcterm) of sequence:

CTTTCTAGTT AAAGATCTTT AATTCACTG

Mutagenesis of the *B.t.t.* toxin gene was performed in pMON9758. A plasmid which contains the new BglII site was designated pMON9787 (Figure 12). Because pMON9787 contains a BglII site just upstream of the ATG initiation codon, the full coding sequence for the *B.t.t.* toxin gene with essentially no 5' or 3' flanking sequence is contained on a BglII fragment of about 1940 bp.

This 1940 bp fragment was isolated from pMON9787 and ligated with BglII digested pMON893. A plasmid in which the 5' end of the *B.t.t.* toxin gene was adjacent to the enhanced CaMV35S promoter was identified and designated pMON9791 (Figure 12).

A variant of the full length *B.t.t.* toxin is produced in *E. coli* from a second methionine initiator codon. This protein, designated "band 3", has been found to be as toxic to Colorado potato beetle as the full length toxin ("band 1"). It is possible

that, as was the case for the B.t.k. gene, truncated forms of the B.t.t. gene might be more easily expressed in plant cells. Therefore, a modified B.t.t. toxin gene was constructed in which the region upstream of the band 3 ATG codon has been removed. In order to remove this sequence, a BglII site was inserted just upstream of the band 3 ATG by the method of Kunkel (1985). The sequence surrounding the band 3 ATG is:

CCAAATCCAACACTAGAAGATTTAAATTATAAAGAGTTTTTAAGAATGACTGCAGATAAT
ProAsnProThrLeuGluAspLeuAsnTyrLysGluPheLeuArgMetThrAlaAspAsn

Mutagenesis was performed with primer (bttnterm) of sequence:

ATCTGCAGTC ATTGTAGATC TCTCTTTATA ATTT

Mutagenesis with this primer was performed on the B.t.t. toxin gene contained in pMON5420. A plasmid containing the new BglII site was designated pMON9788. A truncated B.t.t. toxin gene beginning at this band 3 BglII site and extending to the BglII site just distal to the termination codon found in pMON9787 was constructed in pMON893 as follows. pMON9788 (Figure 13) was digested with BglII and XbaI and a fragment of about 1250 bp was isolated. This fragment extends from the band 3 ATG to a unique XbaI site in the middle of the B.t.t. toxin gene. pMON9787 was also digested with BglII and XbaI, and a fragment of about 550 bp was isolated. This fragment extends from the unique XbaI site in the middle of the toxin gene to the BglII site just distal to the termination codon. These two fragments were mixed and ligated with BglII digested pMON893. A plasmid was identified in which the 5' end to the toxin gene was adjacent to the

enhanced CaMV35S promoter and designated pMON9792. pMON9792 contains a N-terminal truncated derivative of the *B.t.t.* toxin gene (Figure 13) which encodes only band 3.

Both pMON9791 and pMON9792 were introduced into *A. tumefaciens* strain ACO which contains a disarmed Ti plasmid. Cointegrates have been selected and have been used in the transformation of tomato and potato.

ACO is a disarmed strain similar to pTiB6SE described by Fraley et al. (1985). For construction of ACO the starting *Agrobacterium* strain was the strain A208 which contains a nopaline-type Ti plasmid. The Ti plasmid was disarmed in a manner similar to that described by Fraley et al. (1985) so that essentially all of the native T-DNA was removed except for the left border and a few hundred base pairs of T-DNA inside the left border. The remainder of the T-DNA extending to a point just beyond the right border was replaced with a novel piece of DNA including (from left to right) a segment of pBR322, the oriV region from plasmid RK2, and the kanamycin resistance gene from Tn601. The pBR322 and oriV segments are similar to the segments in pMON893 and provide a region of homology for cointegrate formation. The structure of the ACO Ti plasmid is shown in Figure 17.

CHIMERIC *B.t.t.* TOXIN GENE USING A MAS PROMOTER

The MAS promoter was isolated from pTiA6 as a 1.5 kb EcoRI-ClaI fragment. This DNA fragment extends from the ClaI site at nucleotide 20,138 to the EcoRI site at 21,631 in the sequence of Barker et al. (1983). Referring to Figure 15, the EcoRI-ClaI

fragment was ligated with the binary vector pMON505 (Horsch et al. 1986) which had been previously digested with EcoRI and ClaI. The resulting plasmid was designated pMON706. A fragment containing the NOS 3' end was inserted downstream of the MAS promoter to obtain a MAS-NOS 3' expression cassette vector. The NOS 3' fragment was excised from pMON530 as a 300 bp BglII-BamHI fragment and inserted into BglII-digested pMON706. The resulting plasmid was designated pMON707.

Plasmid pMON530 was constructed by cleavage of pMON200 with NdeI to remove a 900 bp NdeI fragment to create pMON503. Plasmid pMON503 was cleaved with HindIII and SmaI and mixed with plasmid pTJS75 (Schmidhauser and Helinski, 1985) that had also been cleaved with HindIII and SmaI. A plasmid that contained the 3.8 kb HindIII-SmaI fragment of pTJS75 joined to the 8 kb HindIII-SmaI fragment of pMON503 was isolated and designated pMON505. Next the CaMV35S-NOS3' cassette was transferred to pMON505 by cleavage of pMON316 with StuI and HindII and isolation of the 2.5 kb StuI-HindIII fragment containing the NOS-NPTII'-NOS marker and the CaMV35S-NOS3' cassette. This was added to pMON505 DNA cleaved with StuI and HindIII. Following ligation and transformation a plasmid carrying the CaMV35S-NOS3' cassette in pMON505 was isolated and designated pMON530.

Since some binary vectors have greatly reduced frequencies of transformation in tomato as compared to co-integrating vectors, (McCormick et al., 1986), the MAS-NOS 3' cassette was moved from pMON707 into the co-integrating vector pMON200 (Fraley et al., 1985). Plasmid pMON200 was digested with StuI and HindIII and a 7.7 kb fragment isolated by agarose gel

electrophoresis. Plasmid pMON707 was similarly digested with StuI and HindIII and a 3.5 kb StuI-HindIII fragment containing the MAS-NOS 3' cassette was isolated by agarose gel electrophoresis and recovery on a DEAE membranes with subsequent elution with 1M NaCl. These two DNA fragments were ligated and the resulting plasmid was designated pMON9741 (Figure 15). This plasmid contains the MAS-NOS 3' cassette in the pMON200 co-integrating background.

Chimeric *B.t.t.* toxin genes driven by the MAS promoter are prepared by digesting either pMON9791 or pMON9792 with BglII, recovering the toxin encoding fragment and moving this fragment into pMON9741 following the teachings provided herein.

These intermediate vectors may be used to transform plants to exhibit toxicity to Coleopteran insects susceptible to the *B.t.t.* toxin protein.

COLEOPTERAN-TYPE TOXIN GENE
EXPRESSION IN PLANTS

Tomato Plant Transformation

The *A. tumefaciens* strains pMON9753-ASE and pMON9754-ASE were used to transform tomato leaf discs by the method of McCormick et al. (1986). Transformed tomato plants were recovered as described and assayed for kanamycin resistance.

Insect Toxicity of Transgenic Tomato Plants

Tomato plants transformed with the *B.t.t.* toxin gene contained in pMON9753 were assayed for expression of the toxin gene by bioassay with Colorado potato beetle (*Leptinotarsa decemlineata*) insects. Leaf cuttings from plants to be assayed were placed in

petri dishes containing water saturated filter paper. Ten or twenty newly hatched potato beetle insects were added to the leaf cuttings and allowed to feed on the leaves. After four days the insects were scored for mortality. In addition, insects were examined for evidence of slowed growth rate (stunting), and the leaf tissue remaining was examined to determine relative feeding damage.

In each experiment many non-transformed plants were included as controls. Between 50 and 100 non-transformed plants have now been assayed as controls. Of these control plants, more than 80% show no mortality to potato beetle; about 15% give 10% mortality; and, 5% or fewer show 20% mortality. Mortality of greater than 20% has not been seen with a control plant.

Table VI below summarizes toxicity results obtained with several pMON9753 transgenic tomato plants.

TABLE VI

Toxicity of Transgenic Tomato Plants Containing
pMON9753 to Colorado Potato Beetle

Plant	Kanamycin[1] Resistance	Mortality of CPB (%)		
		Assay #1	Assay #2	Assay #3
794	R	30	20	
810	n.d.	50	20	40
871	R	30	10 (stunted)	
886	R	50	40	
887	n.d.	20	30	30
1009	n.d.	50		
1044	R	20 (stunted)		
1046	R	40 (stunted)	20	.

[1] n.d. represents No Data

As shown in Table VI several plants have been recovered which consistently show higher levels of mortality of Colorado potato beetle than non-transformed control plants. These results indicate that the *B.t.t.* toxin gene is being expressed at levels sufficient to kill a significant number of the insects feeding on these plants.

COLEOPTERAN TOXIN EXPRESSION IN POTATO

Shoot tips of potato cultivar Kennebec are subcultured on media containing MS major and minor salts, 0.17 g/l sodium dihydrogen phosphate, 0.4 mg/l thiamine-HCl, 0.1 g/l inositol, 3% sucrose, 2.0 g/l Gelrite (Kelco Co.) at pH 5.6. Cultures are grown for 4 weeks at 24°C in a 16 hour photoperiod. Stem internodes are cut into approximately 8mm lengths and the cut surfaces are smeared with *Agrobacterium* strain pMON9753-ASE which has been s⁺reaked on an LB agar plate and grown for 2 to 3 days. pMON9753-ASE which is described above contains the chimeric *B.t.t.* toxin gene driven by the CaMV35S promoter. Alternatively, *Agrobacterium* strains pMON9791-ACO or pMON9792-ACO containing chimeric *B.t.t.* toxin genes are used. Stem sections are placed on 0.8% agar-solidified medium containing salts and organic addenda as in Jarret et al. (1980), 3% sucrose, 3 mg/l BA and 0.1 mg/l NAA at pH 5.6. After 4 days the explants are transferred to medium of the same composition but with carbenicillin at 500 mg/l and kanamycin as the selective agent for transformed plant cells at 100 mg/l. Four weeks later the explants are transferred again to medium of the same composition but with GA_3 at 0.3 mg/l as the sole hormone. Callus which developed in the presence of

100 mg/l kanamycin are shown to contain the NPTII
enzyme when tested by a dot blot assay indicating
that the potato cells are transformed. Uninoculated
control tissue is inhibited at this concentration of
5   kanamycin. Transformed potato tissue expresses the
B.t.t. toxin gene. *B.t.t.* toxin mRNA may be detected
by Northern analysis and *B.t.t.* toxin protein may be
detected by immunoassay such as Western blot analysis.
However, in many cases the most sensitive assay for
10 the presence of *B.t.t.* toxin is the insect bioassay.
Colorado potato beetle larvae feeding on the trans-
formed tissue suffer from the effects of the toxin.

This procedure for producing kanamycin
resistant transformed potato cells has also been suc-
15 cessfully used to regenerate shoots. Shoots which are
1 to 2 cm in length are removed from the explants and
placed on the shoot tip maintenance medium described
above where the shoots readily root.

Plants generated in this fashion are tested
20 for transformation by assaying for expression of the
NPTII enzyme and by the ability of stem segments to
form callus on kanamycin containing medium. Trans-
formed plants express the *B.t.t.* toxin gene. *B.t.t.*
toxin mRNA may be detected by Northern analysis and
25 *B.t.t.* toxin protein may be detected by immunoassay
such as Western blot analysis. Colorado potato beetle
larvae feeding on the transformed tissue suffer from
the effects of the toxin.

COLEOPTERAN TOXIN EXPRESSION IN COTTON

30   Cotton seeds are surface sterilized by first
soaking them for 10 minutes in a detergent solution of
water to which Sparkleen soap has been added, then by

agitating them for 20 min. in a 30% Chlorox solution containing 2 drops of Tween 20 per 400 mls before rinsing them twice with sterile distilled water. The seeds are then soaked in 0.4% benolate for 10 min.

5   The benolate is poured off prior to placing the seeds aspetically onto agar solidified half strength MS salts. Seeds are germinated for 3-10 days in the dark at 32°C. The cotyledons and hypocotyls are then removed aspetically and segmented. The segments are

10   placed onto 1) agar solidified MS medium containing 3% glucose, 2 mg/l napthalene acetic acid (NAA), and 1 mg/l kinetin (Medium MSS) or 2) Gelrite solidified MS medium containing 3% glucose, B5 vitamins, 100 mg/l inositol, 0.75 mg/l $MgCl_2$, 0.1 mg/l dichlorophenoxy

15   acetic acid (2,4-D) and 0.1 or 0.5 mg/l kinetin (Medium MST). Callus is maintained in a 16/8 photo-period at 28°C on either of these media until embryo-genesis is initiated. Subculture of the embryogenic callus is made onto the same medium as for initiation

20   but containing 3% sucrose instead of glucose. Somatic embryos are germinated by moving them onto Gelrite solidified Stewart's medium without plant growth regu-lators but containing 0.75 g/l $MgCl_2$. Germinated embryos are moved to soil in a growth chamber where

25   they continue to grow. Plants are then moved to the greenhouse in order to set seed and flower.

Transformation of cotton tissues and produc-tion of transformed callus and plants is accomplished as follows. Aseptic seedlings are prepared as for

30   plant regeneration. Hypocotyl and cotyledon segments are inoculated with liquid overnight *Agrobacterium* cultures or with *Agrobacterium* grown on nutrient plates. The explants are co-cultured for 2-3 days on MSS or MST medium containing 1/10 the concentration

of MS salts. Explants are blotted on filter paper to remove excess bacteria and plated on MSS or MSN medium containing 500 mg/l carbenicillin amd 30-100 mg/l kanamycin. Callus which is transformed will grow on

5   this medium and produce embryos. The embryos are grown into plants as stated for regeneration. The plants are tested for transformation by assay for expression of NPTII.

When the Agrobacterium strain used for

10 transformation contains a chimeric B.t.t. toxin gene such as pMON9753, pMON9791 or pMON9792, the B.t.t. toxin gene is expressed in the transformed callus, embryos derived from this callus, and in the trans- formed plants derived from the embryos. For all of

15 these cases, expression of the B.t.t. toxin mRNA may be detected by Northern analysis, and expression of the B.t.t. toxin protein may be detected by immuno- assay such as Western blot analysis. Insect bioassay may be the most sensitive measure for the presence of

20 toxin protein.

Insect toxicity of the callus, embryos or plants is assayed by bioassay with boll weevil larvae (Anthonomous grandis). Boll weevil larvae feeding on transformed cotton cells or plants expressing the

25 B.t.t. toxin gene suffer from the effects of the toxin.

COLEOPTERAN TOXIN GENE EXPRESSION IN MAIZE

The following description outlines the pre- paration of protoplasts from maize, the introduction

30 of chimeric B.t.t. toxin genes into the protoplast by electroporation, and the recovery of stably trans- formed, kanamycin resistant maize cells expressing chimeric B.t.t. toxin genes.

Preparation of Maize Protoplasts

Protoplasts are prepared from a Black Mexican Sweet (BMS) maize suspension line, BMSI (ATCC 54022) as described by Fromm et al. (1985 and 1986).

5   BMSI suspension cells are grown in BMS medium which contains MS salts, 20 g/l sucrose, 2 mg/l (2,4-dichlorophenoxy) acetic acid, 200 mg/l inositol, 130 mg/l asparageine, 1.3 mg/l niacin, 0.25 mg/l thiamine, 0.25 mg/l pyridoxine, 0.25 mg/l calcium pantothenate, pH

10   5.8. Forty ml cultures in 125 ml erlenmeyer flasks are shaken at 150 rpm at 26°C. The culture is diluted with an equal volume of fresh medium every 3 days. Protoplasts are isolated from actively growing cells 1 to 2 days after adding fresh medium. For proto-

15   plast isolation cells are pelleted at 200 X g in a swinging bucket table top centrifuge. The supernatant is saved as conditioned medium for culturing the protoplasts. Six ml of packed cells are resuspended in 40 ml of 0.2 M mannitol/50 mM $CaCl_2$/10 mM sodium acetate

20   which contains 1% cellulase, 0.5% hemicellulase and 0.02% pectinase. After incubation for 2 hours at 26°C, protoplasts are separated by filtration through a 60 μm nylon mesh screen, centrigured at 200 X g, and washed once in the same solution without enzymes.

25   ## Transformation of Maize Protoplasts with
B. t. t. Toxin Gene DNA Vectors Using an
Electroporation Technique

Protoplasts are prepared for electroporation by washing in a solution containing 2 mM potassium

30   phosphate pH 7.1, 4 mM calcium chloride, 140 mM sodium chloride and 0.2 M mannitol. After washing, the protoplasts are resuspended in the same solution at a concentration of 4 X 10^6 protoplasts per ml. One-half

ml of the protoplast containing solution is mixed with 0.5 ml of the same solution containing 50 micrograms of supercoiled plasmid vector DNA and placed in a 1 ml electroporation cuvette. Electroporation is carried out as described by Fromm et al. (1986). As described, an electrical pulse is delivered from a 122 or 245 microFarad capacitor charged to 200 V. After 10 min. at 4°C and 10 min. at room temperature protoplasts are diluted with 8 ml of medium containing MS salts 0.3 M mannitol, 2% sucrose, 2 mg/l 2,4-D, 20% conditioned BMS medium (see above) and 0.1% low melting agarose. After 2 weeks in the dark at 26°C, medium without mannitol and containing kanamycin is added to give a final kanamycin concentration of 100 mg/l liquid. After an additional 2 weeks, microcalli are removed from the liquid and placed on a membrane filter disk above agarose solidified medium containing 100 mg/l kanamycin. Kanamycin resistant calli composed of transformed maize cells appear after about 1-2 weeks.

Expression of *B.t.t* Toxin Genes in Maize Cells

As described by Fromm et al. (1986), transformed maize cells can be selected by growth in kanamycin containing medium following electroporation with DNA vectors containing chimeric kanamycin resistance genes composed of the CaMV35S promoter, the NPTII coding region and the NOS 3' end. pMON9791 and pMON9792 contain such chimeric NPTII genes and also contain chimeric *B.t.t.* toxin genes. As decribed above, maize protoplasts are transformed by electroporation with DNA vectors where the DNA vectors are pMON9791 or pMON9792. Following selection for kanamycin resistance, the transformed maize cells are assayed for expression of the *B.t.t.* toxin gene.

Assays are performed for *B.t.t.* mRNA by Northern blot analysis and for *B.t.t.* toxin protein by immunoassay such as Western blot analysis.

   Assays for insect toxicity are performed by feeding transformed maize calli to Southern corn rootworm larvae (*Diabrotica undecimpunctata howardi*). Alternatively, a protein extract containing the *B.t.t.* toxin protein is prepared from transformed maize cells and this extract is incorporated into an appropriate insect diet which is fed to the Southern corn rootworm larvae. Rootworm larvae feeding on transformed calli or protein extracts of such calli suffer from the effects of the toxin.

   The above examples are provided to better elucidate the practice of the present invention and are not intended, in any way, to limit the scope of the present invention. Those skilled in the art will recognize that modifications may be made without deviating from the spirit and scope of the invention as described.

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